Application of Competitive Enzyme-Linked Immunosorbent Assay for the Quantification of Imidacloprid Titers in Xylem Fluid Extracted from Grapevines

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ABSTRACT A competitive enzyme-linked immunosorbent assay (ELISA) technique was evaluated for quantifying titers of imidacloprid in xylem fluid extracted from *Vitis vinifera* L. grapevines that were treated with systemic applications of the neonicotinoid insecticide Admire. Evidence of matrix effects, factors that compromise the precision and accuracy of the ELISA, was present in assays with undiluted xylem fluid. These effects could be eliminated by dilution of extracts in water, resulting in a lower sensitivity of the assay of 4 μg liter^{-1}. In a field trial conducted in a commercial vineyard, there was an excellent correlation between Admire application rates and xylem fluid concentrations of imidacloprid. At an Admire application rate of 1.17 liter ha^{-1} (16 fl oz per acre), uptake of imidacloprid into vines was rapid. Imidacloprid was consistently detected in the xylem for up to 3 mo after application at concentrations known to be effective at managing populations of the sharpshooter *Homalodisca coagulata* Say, an important vector of *Xylella fastidiosa* Wells in California vineyards. The ELISA is a sensitive technique that can be used to study the behavior of systemic insecticides within crop systems and their impact on pest populations.

KEY WORDS imidacloprid, ELISA, xylem, grapevine, *Homalodisca coagulata*

IMMUNOASSAYS ARE WIDELY USED for qualitative and quantitative assessments of environmental samples for the presence of contaminants such as pesticides and are often favored over other methods such as gas chromatography, high-performance liquid chromatography, and mass spectrometry because of their speed, sensitivity, and cost-effectiveness (Hammock and Mumma 1980). Although many immunoassays are designed specifically for environmental monitoring purposes, there is the additional possibility that established immunoassays can be used to provide a better understanding of the behavior of an insecticide, in terms of its uptake and persistence, after its application to a crop. This information would greatly benefit pest management if it enables the establishment of threshold levels of insecticide required to protect plants from pest infestation. Studies to better understand the behavior of insecticides within agricultural environments are restricted by the lack of simple, yet effective, analytical tools. Immunoassays are a very attractive option in part because of their ability to deal with large sample sizes generated from extensive monitoring programs. This advantage of immunoassays was demonstrated most emphatically in a recent study conducted on citrus trees in southern California (Castle et al. 2005), in which a competitive enzyme-linked immunosorbent assay (ELISA) technique was used to monitor titers of the neonicotinoid insecticide 1-(6-chloro-3-pyridinylmethyl)-*N*-nitro-2-imidazolidinimine (imidacloprid) in trees that were treated with Admire, the systemic formulation of imidacloprid. The use of the ELISA helped to establish the dynamics of imidacloprid uptake and its distribution within citrus trees and the titers of imidacloprid that were necessary to effectively suppress densities of the recently introduced pest, the sharpshooter *Homalodisca coagulata* Say. *H. coagulata* has become the principal vector of the plant pathogenic, xylem-limited bacterium *Xylella fastidiosa* Wells in southern California (Costa et al. 2000) since the introduction of the insect into the state in the early 1990s (Blua et al. 1999). Strains of *X. fastidiosa* cause several economically important plant diseases, including Pierce's disease in *Vitis vinifera* L. grapevines. In the Temecula Valley viticultural region, which consists of ≈700 ha of wine grapes, a severe Pierce's disease epidemic that began in 1996 resulted in some wineries reporting losses of 20 to 30% of their vines (Hix 2001). There is no cure for Pierce's disease and infected vines must be removed from the vineyard to reduce the risk of *H. coagulata* becoming infective from feeding on them and transferring the pathogen to healthy vines. Insecticides are currently the most effective means of managing *H. coagulata* populations in vineyards. In particular, the systemic behavior of imidacloprid is well suited to

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exploit the xylophagous feeding behavior of *H. coagulata*, and this insecticide is now widely used in vineyards (Castle et al. 2005).

The purpose of this study was to evaluate the effectiveness of a competitive ELISA technique, available commercially for residue analysis of environmental water samples, for quantifying imidacloprid titers in xylem fluid extracted from grapevines treated with Admire. Matrix effects can be particularly problematic in ELISAs. These effects occur when components in the test matrix (the xylem fluid) disrupt the interaction between the antibody and the target molecule (imidacloprid). Experiments were initially designed to evaluate potential matrix effects attributable to xylem fluid and the degree of sensitivity of the assay. Subsequent field experiments were then conducted in a commercial vineyard, by using the ELISA to quantify imidacloprid titers in grapevines, with the aim of establishing its rate of uptake and temporal persistence within treated vines.

Materials and Methods

Vineyard Study Site. Field studies were conducted during the 2002 and 2003 grape seasons at a commercial vineyard in the Temecula Valley of southern California. The vineyard was newly established in 2002 and consisted of 2 ha of Sirah vines. The soil composition, determined from 10 randomly selected soil cores (to a 15-cm depth) within the study area, was characterized by the University of California's Division of Agricultural and Natural Resources Analytical Laboratory as a loamy sand (86% sand, 7% silt, and 7% clay) with a pH of 6.5 and an average organic matter content of 0.7%. During our study, vines were watered once weekly for 4 h by drip irrigation that administered water directly to the root zone of each vine.

Insecticide Applications. Admire (240 g liter^{-1} Flowable Insecticide; Bayer CropScience, Research Triangle Park, NC) was applied by chemigation, by using the drip irrigation system established at the vineyard. In accordance with label recommendations, vines were pre-irrigated for at least 1 h before the application of insecticide to ensure adequate wetting of the soil. In 2002, Admire was applied at 1.17, 1.46, and 2.34 liter ha^{-1} to three separate 0.08-ha blocks of vines (comprising 150 vines each) by injection into the irrigation lines. After injection, the irrigation lines were flushed with water for 2 h. Thereafter, the irrigation was run weekly in accordance with the grower's established agronomic practices. In 2003, Admire was applied to the same site at a single rate of 1.17 liter ha^{-1}.

Xylem Fluid Extraction. Xylem fluid was extracted from grape stems by using a plant water stress console (pressure chamber), an instrument that is normally used for measuring the water potential of plants (Scholander et al. 1965). The first samples of xylem fluid were extracted immediately before the application of Admire, and thereafter at least once every 2 wk over a 4-mo sampling period. Sampling frequency was most intensive immediately after the Admire application, to establish the initial rate of uptake. Xylem fluid was extracted from cane terminals cut from 16 treated vines on each sampling date. The outer ring (2.5 cm in length) of phloem tissue was stripped from the stem at the severed end to minimize phloem content during extractions and to maximize xylem fluid content. Xylem fluid extracts were obtained which were largely devoid of phloem and other tissue contents, thereby giving a better estimate of the mobile imidacloprid component within the xylem vascular system. Once the stem was inserted through the grommet in the sealing lid, the pressure chamber was pressurized to 35 kg/cm^2 (500 psi) delivered from a cylinder of compressed air. At this pressure, the xylem fluid was extruded easily from the stem and was collected using a 1-ml pipette. Typically, a minimum of 200 μl was collected from each stem, and the fluid was then added to a 1.5-ml Eppendorf tube. Samples were immediately transferred to dry ice for storage.

Chemical Quantification. Concentrations of imidacloprid within xylem fluid samples were determined using a competitive ELISA technique, in which insecticide residues in the xylem fluid extracts compete with enzyme (horseradish peroxidase)-labeled imidacloprid for a limited number of antibody binding sites on the wells of a microplate. The levels of bound conjugate are determined colorimetrically and are inversely proportional to the levels of insecticide present in the xylem fluid. The ELISA kit is available commercially (QuantiPlate kit for imidacloprid, catalog no. EP 006, EnviroLogix Inc., Portland, ME) and has a reported 0.2–6 μg imidacloprid liter^{-1} sensitivity range. The assay was calibrated in our laboratory before use to test for matrix effects associated with xylem fluid. To ensure that preliminary biochemical evaluations of the ELISA were not compromised by imidacloprid contamination, xylem fluid was extracted from vines in an organically managed vineyard in Temecula that had never been treated with imidacloprid.

The first series of tests involved the dilution of a 5 μg imidacloprid liter^{-1} standard calibrator (supplied with the kit) in either water or 100% xylem fluid to give concentrations of 0.2, 0.5, 1, and 2 μg imidacloprid liter^{-1}. Under these conditions, the imidacloprid concentration was decreased as the proportion of xylem fluid within the mixtures was increased. Additional tests were performed to measure the recovery of imidacloprid from spiked xylem fluid samples. For this, a range of xylem fluid concentrations was initially prepared by diluting several extracts with water, and then mixing each with an equal volume of imidacloprid to give a final concentration of 0.2 μg imidacloprid liter^{-1} (the sensitivity limit of the assay kit as supplied). Based on results from these preliminary experiments, standard curves for imidacloprid were then constructed for water and 5% xylem fluid. A series of imidacloprid concentrations were prepared in water, and to each concentration was added an equal volume of 10% xylem fluid to give the desired imidacloprid concentration range in a constant 5% xylem fluid background. All water stock solutions of imidacloprid were

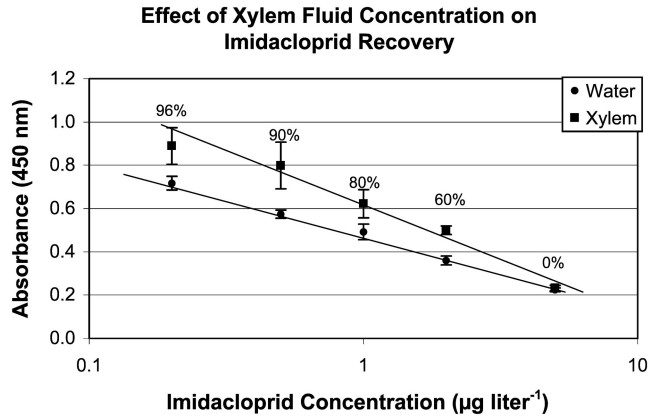

Fig. 1. Effect of xylem fluid concentration on the detection of imidacloprid by ELISA. A 5 μg imidacloprid liter^{-1} stock was diluted with either water or xylem extract, resulting in an increased extract concentration with successive dilutions of the standard. The numbers adjacent to each point on the xylem series indicate the percentage of xylem fluid tested at that specific imidacloprid concentration. Each data point represents the mean ± SE of three independent assessments.

freshly prepared immediately before each experiment.

Statistical Analysis. All statistical analyses were performed using the GraphPad Prism version 4 (GraphPad Software, Inc., San Diego, CA). Analysis of variance (ANOVA) was used to test for significant effects of xylem fluid concentration on imidacloprid recovery, whereas pairwise comparisons of means were analyzed by Tukey's honestly significant difference (HSD) test. Linear regression was used to determine the relationship between application rate and xylem fluid content. The significance level for all statistical tests was set at $P = 0.05$.

Results

Evidence of Matrix Effects. Matrix effects associated with grape xylem fluid were detected using several protocols (Figs. 1–3). Dilution of the 5 μg imidacloprid liter^{-1} calibrator with water and xylem fluid yielded significantly different responses ($F = 22.70$; df = 1, 21; $P = 0.0001$; Fig. 1). This indicated that matrix effects occurred over the sensitivity range of the assay when xylem fluid concentrations were at least 60%. Mild effects also were detected at lower xylem fluid concentrations based on measurements of the recovery of 0.2 μg imidacloprid liter^{-1} (Fig. 2). Although these mild effects were not significant ($F = 1.72$; df = 5, 18; $P = 0.182$), the noticeable increase in absorbance at 10% xylem fluid was of concern. All evidence of matrix effects was eliminated when the fluid was diluted at least 20-fold in water. The adequacy of this dilution step was confirmed by comparing standard curves for imidacloprid prepared in 5% xylem fluid and water ($F = 0.57$; df = 1, 26; $P = 0.46$; Fig. 3). Our experiments thus established that the

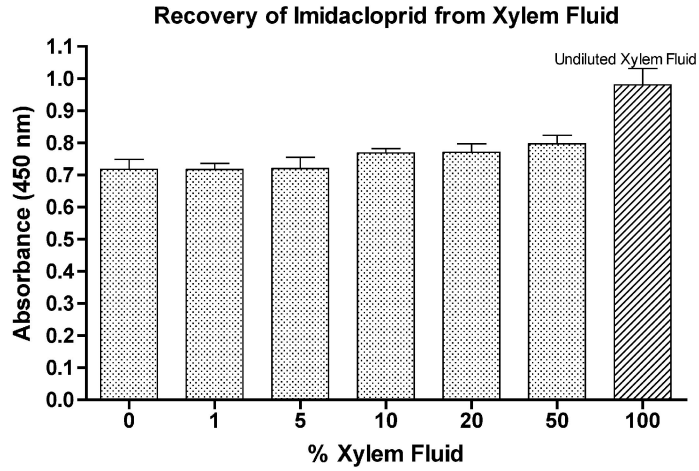

Fig. 2. Impact of xylem fluid concentration on the detection of 0.2 μg imidacloprid liter^{-1} by ELISA. Matrix effects were eliminated when the extracts were diluted at 20-fold with water before assay. The undiluted xylem extract contained no imidacloprid and was included to show the response in assays of unadulterated xylem fluid. Each bar represents the mean ± SE of four replicate extracts.

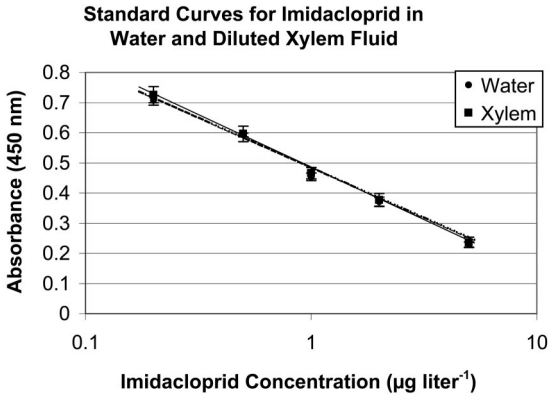

Fig. 3. Standard curves for imidacloprid prepared in water, and xylem fluid that was diluted 20-fold with water before ELISA. Each point represents the mean ± SE of a minimum of three independent determinations.

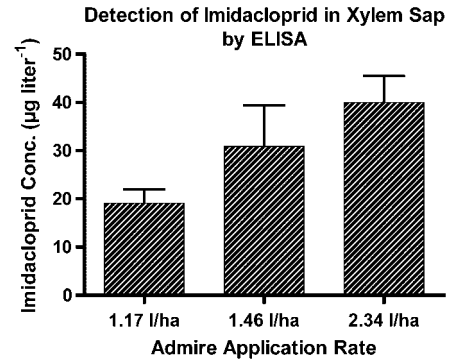

Fig. 4. Impact of Admire application rate on imidacloprid concentration measured within xylem fluid of grapevines. Xylem fluid was extracted from vines two months after the treatment. Each bar represents the mean ± SE of 10 vines chosen randomly within each treatment block.

binding characteristics of standards prepared in water were comparable with a series of standards prepared directly in xylem fluid, provided that the fluid was diluted at least 20-fold before conducting the assay (Fig. 3). The linear range of detection in grape xylem fluid was thus 4–120 μg liter^{-1} for imidacloprid. For sample analyses, extracts were routinely diluted 20-fold before assays, and when readings measured above the detection limits of the assay, the samples were diluted further in distilled water and the assay rerun. Assays were conducted in accordance with the kit recommendations.

Field Trials. The first experiment to evaluate the ELISA under field conditions was conducted in a newly established vineyard of Sirah grapes that was treated with three rates of Admire. Due to the age of the vines, and the destructive nature of the sampling process, sampling during the first season was limited to just one date at two months post-application. The Admire application rate had a significant impact upon the titers of imidacloprid within the xylem fluid (F = 7.31; df = 2, 27; P = 0.003; Fig. 4). The two-fold increase in application rate between the 1.17 and 2.34 liter ha^{-1} rates resulted in a doubling of imidacloprid titers, whereas the 1.46 liter ha^{-1} rate resulted in intermediate levels (Fig. 4).

During 2003, a more substantial sampling program was conducted over a 4-mo period within the same vineyard, using a single Admire application of 1.17 liter ha^{-1} (Fig. 5). Imidacloprid was detected within the vines on 23 June, 3 d post-treatment, and quickly rose to >40 μg imidacloprid liter^{-1} by day 5. Peak levels of imidacloprid were measured on 16 July, 26 d after application. During the entire 15-wk sampling period, the average titers on any sampling date remained above 15 μg imidacloprid liter^{-1}.

Discussion

Xylem is the principal water-conducting tissue of vascular plants and the primary means of distribution for soil-applied systemic insecticides such as imidacloprid (Elbert et al. 1990). Therefore, the availability of a tool for the detection and quantification of residues within xylem fluid could provide pest management specialists with valuable insights into the dynamics of uptake and availability of systemic insecticides. In this study, we have shown the utility of a competitive ELISA technique for quantifying imidacloprid titers within the xylem fluid of grapevines after their systemic treatment with Admire. With appropriate calibration before sample analysis, the assay proved to be very effective at determining insecticide levels within the xylem, with a lower limit of quantification of 4 μg imidacloprid liter^{-1}.

In the initial field assessment of the ELISA, there was an excellent correlation between the rate of Admire application and the resulting titers measured within the xylem fluid. This outcome provides strong evidence for the suitability of the ELISA technique for monitoring imidacloprid titers within xylem fluid and

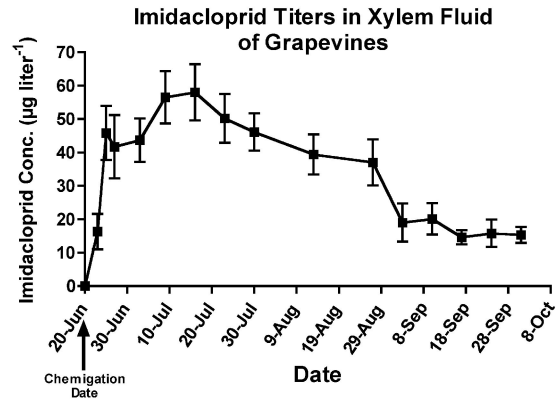

Fig. 5. Profile of imidacloprid concentrations in grapevines treated systemically with Admire at a rate of 1.17 liter ha^{-1} (16 fl oz per acre) on 20 June 2003 (indicated by the arrow). Each point represents the mean ± SE of 16 vines. The same vines were sampled throughout the assessment period.

for assessing the impact of application rates on these titers. More extensive sampling was undertaken in the 2003 field trial. Levels of imidacloprid measured within the xylem of treated vines rose rapidly after chemigation with 1.17 liter ha^{-1} Admire and were maintained above 15 μg imidacloprid $liter^{-1}$ for at least 3 mo. In a recent study, in which the ELISA was used to monitor imidacloprid titers in xylem fluid extracted from citrus trees treated with 2.34 liter ha^{-1} Admire, populations of *H. coagulata* began to decline and remain at low levels when the imidacloprid concentrations were at least 10 μg $liter^{-1}$ (Castle et al. 2005). In our study, we used 1.17 liter ha^{-1} and still maintained concentrations of imidacloprid above this minimum effective level. This information is encouraging for growers in their efforts to protect their vines from *H. coagulata* infestations. Our results highlight the utility of the assay as a pest management tool that is capable of monitoring pesticide titers in crops to determine whether they are at acceptable levels for effective pest control. With appropriate calibration, the ELISA will be an ideal tool for assessing the behavior of imidacloprid within different plant systems. It will be especially useful for establishing suitable application rates on plants such as grapevines.

After application of Admire, imidacloprid is continuously taken into the roots over a prolonged period and distributed through the xylem tissue to all vegetative parts of the plant (Elbert et al. 1990). The assay is, therefore, ideally suited for measurements of insecticide concentrations within the rather innocuous background of xylem fluid. However, extra care is required when using similar assays to quantify residues in extracts that are likely to contain the contents of other plant tissues. One potential problem is the likelihood that metabolites of the parent compound will cross-react with the antibody. The metabolism of imidacloprid within plants has been well documented (Westwood et al. 1998; Nauen et al. 1998, 1999), and several of the metabolites are known to cross-react with antibodies that have been prepared for the detection of imidacloprid (Li and Li 2000, Lee et al. 2001, Wanatabe et al. 2001). For this reason, the competitive ELISA approach may be best suited to imidacloprid quantification during its mobilization within the xylem before it encounters plant detoxification systems. To optimize xylem content in extracts, we recommend using the pressure bomb as an extraction device. The isolation of the xylem before fluid extraction provided for a more concentrated plant extract focused on the xylem contents and eliminated the necessity for postextraction cleanup. After extraction, samples were ready for analysis and required nothing more than simple dilution in water to mitigate relatively mild matrix effects.

There is an increasing number of neonicotinoid insecticides under development or testing for systemic use. Although the relative efficacies of these materials against specific insect pests can be assessed using bioassays, their toxicological profiles may not reflect their efficacy under field conditions, due to differences in uptake caused by agronomic or abiotic factors (e.g., irrigation practices, and soil type) that impact delivery to the pest's feeding zone. We are currently using the ELISA approach in field studies that are aimed at evaluating the uptake and persistence of toxicologically active chemicals under various agronomic and environmental conditions. This approach will contribute important information for improving the deployment of insecticides within different cropping systems. Although it is unlikely that growers will have the facility to perform assays themselves, the ELISA technique could assist researchers in their studies on the behavior of imidacloprid and other neonicotinoids for which assays are available, within agricultural environments. The information obtained from such studies can then be used to evaluate the suitability of systemic insecticide treatments across the diverse array of agronomic and environmental conditions characteristic of California agriculture.

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